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**Débora de Oliveira  
Reis**

**Caracterização do potencial citotóxico das nanoplaquetas  
de grafeno em células do pulmão**

**Characterization of the cytotoxic potential of graphene  
nanoplatelets in lung cells**

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**Débora de Oliveira  
Reis**

## **Caracterização do potencial citotóxico das nanoplaquetas de grafeno em células do pulmão**

### **Characterization of the cytotoxic potential of graphene nanoplatelets in lung cells**

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Helena Cristina Correia de Oliveira, Investigadora em Pós-Doutoramento do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

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Dedico este trabalho aos meus pais, por todos os sacrifícios que fizeram para que pudesse seguir os meus sonhos e por nunca deixarem de acreditar em mim.

## **O júri**

Presidente

Professora Doutora Maria Helena Abreu Silva  
Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

Arguente Principal

Doutora Sónia Marina Pinto Nunes da Silva  
Investigadora em Pós-Doutoramento do Departamento de Química, QOPNA e CESAM da Universidade de Aveiro

Orientadora

Doutora Helena Cristina Correia de Oliveira  
Investigadora em Pós-Doutoramento do Departamento de Biologia e CESAM da Universidade de Aveiro

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## palavras-chave

Grafeno, nanoplaquetas de grafeno, linha celular A549, toxicidade, ciclo celular, Espécies Reactivas de Oxigênio

## resumo

O grafeno representa um dos alótropos de carbono. As suas características físico-químicas conferem-lhe propriedades únicas: elevada área superficial, condutividades elétrica e térmica excepcionais, grande resistência mecânica, capacidade de bio-funcionalização e produção em massa. Todas estas qualidades tornam o grafeno amplamente aplicável e por isso é necessário investigar detalhadamente os seus possíveis efeitos adversos no contexto da saúde humana. Neste estudo foi avaliado o potencial citotóxico das nanoplaquetas de grafeno em células do pulmão (linha celular A549). As células foram cultivadas *in vitro* e expostas a várias concentrações de grafeno na forma de nanoplaquetas (5, 10, 25, 50, 100 µg/ml) durante 24h. A viabilidade celular, analisada pelo ensaio WST-8, não diminuiu com a exposição às nanoplaquetas de grafeno, tendo, porém, sido verificado um aumento da proliferação celular para a dose mais baixa. A produção de ROS, bem como a dinâmica do ciclo celular das células A549 também não foram afetados pela exposição às nanoplaquetas de grafeno. Os estudos da internalização celular das nanoplaquetas de grafeno por variação da dispersão lateral da luz mostraram que para a concentração mais elevada (100µg/ml) ocorreu um aumento significativo da internalização destas partículas. Por último, não se verificou indução de apoptose nas células A549 após 24h de exposição às nanoplaquetas de grafeno. De uma forma geral, estes resultados sugerem que as nanoplaquetas de grafeno não induzem toxicidade na linha celular A549.

**keywords**

Graphene, graphene nanoplatelets, cell line A549, toxicity, cell cycle, reactive oxygen species

**abstract**

Graphene is one of the carbon allotropes. Its physical and chemical characteristics provide unique properties: high surface area, exceptional electrical and thermal conductivities, high mechanical strength, ability to bio-functionalization and mass production. All these qualities make graphene widely applicable and, therefore, it is necessary to thoroughly investigate their possible adverse effects in the context of human health. This study evaluated the cytotoxic potential of graphene nanoplatelets in lung cells (cell line A549). The cells were cultured *in vitro* and exposed to various concentrations of graphene nanoplatelets (5, 10, 25, 50, 100µg/ml) for 24h. Cell viability, assessed by WST-8 assay, did not decrease after exposure to graphene nanoplatelets, however, an increase in cell proliferation for the lowest concentration was observed. ROS production as well as cell cycle dynamics of A549 cells were not affected by graphene nanoplatelets exposure. Cellular uptake of the graphene nanoplatelets, evaluated by changes in light side scatter by flow cytometry, increased significantly for the highest concentration (100µg/ml). In addition, there was no induction of apoptosis in A549 cells after 24 hours of exposure to the graphene nanoplatelets. Overall, these results suggest that graphene nanoplatelets do not induce toxicity in cell line A549.



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## List of Acronyms and Abbreviations

A549	Cancerous human lung cell line
AFM	Atomic force microscopy
BEAS-2B	Normal human lung (bronchus) cell line
BET	Brunauer-Emmett-Teller
C2C12	Mouse mesenchymal progenitor cells
CNTs	Carbon nanotubes
CXYG	Carboxyl graphene nanoplatelets
DCF	2'-7'-dichlorofluorescein
DCFH-DA	2'-7'-dichlorodihydrofluorescein diacetate
Dh	Hydrodynamic diameter
DLS	Dynamic light scattering
EDTA	Ethylenediaminetetraacetic acid
F-12K	Kaighn's Modification of Ham's F-12 Medium
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FLG	Few-layer graphene
FS	Forward-scatter
GFN	Graphene-Family Nanomaterials
GQDs	Graphene Quantum Dots
GO	Graphene Oxide
GN	Graphene Nanosheets
GNP	Graphene Nanoplatelets
GP	Graphene Platelets
HeLa	Cancerous human cervix cell line
hNSCs	Human Neural Stem Cells
LDH	Lactate dehydrogenase
MBE	Molecular Beam Epitaxy
MLG	Multi-layer graphene
MTT	[3-(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide]
NADH	Reduced Nicotinamide Adenine Dinucleotide
NMs	Nanomaterials
NPs	Nanoparticles
OD	Optical Density
p-53	tumor suppressor protein
PBS	Phosphate buffer saline
PC12	Pheochromocytoma of the rat adrenal medulla cell line
PCGO	Protein-coated graphene oxide
PEG	Polyethylene glycol
PI	Propidium iodide

PLHC-1	<i>Poeciliopsis lucida</i> hepatocellular carcinoma cell line
PS	Phosphatidylserine
rGO	reduced-Graphene Oxide
ROS	Reactive Oxygen Species
RPMI 1640	Roswell Park Memorial Institute 1640 medium
RPMI 8226	Human multiple myeloma cells
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SEM	Scanning electron microscopy
SS	Side-scatter
SWCNTs	Single-wall carbon nanotubes
TEM	Transmission electron microscopy
UK DIUS	United Kingdom Department for Innovation, Universities and Skills
U87	Human primary glioblastoma cell line (U-87 MG - Uppsala 87 Malignant Glioma line)
U118	Human Brain Glioma Cells (U-118 MG - Uppsala 118 Malignant Glioma line)
WST-8	[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]

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## **1. Introduction**

### ***1.1. Historical Overview***

Nanotechnology is a science that works on the design, synthesis, development, fabrication and characterization of materials at the nanoscale (Venugopal et al. 2016). It includes areas such as physics, chemistry, biology, material science, health sciences and engineering (Nikam et al. 2014).

The interest on nanomaterials started a long time ago, when Richard Zsigmondy proposed the concept of “nanometer”, having won the 1925 Nobel Prize Laureate in Chemistry (Hulla et al. 2015). After this, the term “nanotechnology” emerged in 1959, when a physicist called Richard Feynman spoke at an American Physical Society meeting (Tarafdar et al. 2013). In this meeting, he presented a lecture titled “There’s plenty of room at the bottom”, introducing the idea of manipulating matter at the atomic level (Hulla et al. 2015). In 1974, Norio Taniguchi, from the Tokyo University of Science, defined “nanotechnology” as a technique that consists in separation, consolidation and deformation of elements by one atom or by one molecule (Tarafdar et al. 2013). Later, in the 1980s, K. Eric Drexler contributed to the development of the nanoscale phenomena through speeches and books (Tarafdar et al. 2013). Since that, the interest in the fields of nanoscience and nanotechnology has been increasing exponentially, with global investments in research and a high number of patents filing related to the technology and products developed within this field (Tarafdar et al. 2013).

### ***1.2. Applications of Nanotechnology***

Nanotechnology is an area that is constantly developing. With a high commercial and academic interest, new studies arise every day that seek to apply the extraordinary features of nanomaterials in our daily life.

Nanotechnology can be applied for example to renewable energy, avoiding crises that may result from the exhaustion of fossil fuels (Salamanca-Buentello et al. 2016). The use of photovoltaic technology to capture solar energy (solar cells) is presented as a form of energy sustainability where photovoltaic nanostructures are applied (Mao et al. 2007). Most nanotechnology applications are related to human health care. The demand for

cheaper and effective medical treatments has led to extensive research and to the development of new technologies. Nanomedicine has the function to improve methods and bring technological advances so that health care services can be more efficient (Chakraborty et al. 2011). It gives the opportunity to better understand how human body works and provides tools to fight diseases like cancer. This is a field in expansion and it seeks to respond to the challenges posed by medical science, with special focus on diagnosis, drug delivery systems and patient care (Sahoo et al. 2007). Agriculture is another domain in which nanotechnology can be applied to the development of new tools for the detection and treatment of diseases and the improvement of plants in their efficiency to absorb nutrients (Rai et al. 2012). The use of nanomaterials can help solve problems like pests and improve insecticides and pesticides (Rai et al. 2012). The use of nanotechnology in the food sector still raises some concerns from consumers. However it can be applied, for example, in the packaging of goods, constituting protective barriers against gases and bacteria; the use of nanosensors for the detection of contaminants is another strategy for enhancing foods (Duncan 2011). Access to potable water has become a concerning issue, mostly in less developed countries. The employment of membranes and filters based on carbon nanotubes, nanoporous ceramics, magnetic nanoparticles and other nanomaterials, has already been implemented for the removal of contaminants in water (Hillie et al. 2007). Nanoremediation is a field of nanoscience that attempts to solve problems related to the environmental rehabilitation, applying reactive nanomaterials for transformation and detoxification of pollutants (Karn et al. 2009).

Man has always been exposed to nanoparticles (NPs) in the air, however with the industrial revolution this exposure increased due to the wide use of thermal degradation sources, such as power stations and internal combustion engines (Oberdörster et al. 2005). With the appearance and development of nanotechnology, exposure to NPs have increased, either by inhalation, ingestion, skin contact or injection. The NPs have unique physical, chemical and mechanical characteristics, which give them a high potential for numerous applications. However, these same properties, which apparently are ideal for use in industry and medicine, are likely to be harmful when they interact with cells.

In addition to NPs found in the air, some workplaces are also capable of produce them in quantities which can range up to several hundred micrograms per cubic meter (Oberdörster et al. 2005). Inhalation is the major route of exposure, still it is necessary to take into

account the intake and skin contact during manufacture or use of these nanomaterials. Over the years and due to the expansion of these nanomaterials, the interest their potential side effects on human health has increased.

Nowadays, the nanomaterials play a major role in the economy, either by their values and their applications. They have a wide range of applications: solar panels, catalysts, tumor therapies, electronics and batteries. One of the major aims of the utilization of nanomaterials is the improvement of quality of life by refining existing materials and products.

### ***1.3. Nano – words definitions***

Nanomaterials can be divided into two major classes: natural and anthropogenic nanomaterials (Dhawan et al. 2010). Natural nanomaterials exist in Nature, for example as soil colloids (clay minerals, metal oxides, etc.), crystals of sea salt, and others. The anthropogenic nanomaterials can be further classified as incidental (nanomaterials manmade unintentionally – road and air transports) and as manufactured (nanomaterials produced because of their properties) (Dhawan et al. 2010).

The term nanomaterial does not have a single definition (Boholm et al. 2016). To the scientific research, definitions are very important since they allow replication and precise critical evaluation (Boholm et al. 2016).

Many definitions have been proposed, for example, the United Kingdom Department for Innovation, Universities and Skills (UK DIUS), defines a nanomaterial as “a material having one or more external dimensions in the nanoscale or which is nanostructured” (British Standards Institution 2007); the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) describes a nanomaterial as “any form of material that is composed of discrete functional parts, many of which have one or more dimensions of the order of 100 nm or less” (SCENIHR 2007); the European Commission adopted a definition of a nanomaterial in 2011 (Potočník 2011) that characterizes a nanomaterial “as a natural, incidental or manufactured material, containing particles in the unbound state or as an aggregate or an agglomerate, and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1nm – 100nm”.



In addition to nanomaterial, there are other important terms regarding to nanotechnology (Khanna 2016):

- Nano-object – it is a common term applied to all distinct nanoscale objects; it refers to an object with one or more dimensions in the nanoscale.
- Nanoparticle – it is basically a nano-object with its three dimensions in the nanoscale.
- Nanoplate – it has one dimension in the nanoscale, but the other two are much wider.
- Nanofiber – it has two dimensions in the nanoscale and the third dimension is wider than the first.
- Nanotube – it can be considered as an unfilled nanofiber.

There is some controversy about the nano-words and the absence of a single definition can be a problem for the scientific progress and regulation (Boholm et al. 2016); however, it is important to point that the manipulation of the nanomaterials is covered by laws to ensure safety and health to workers and protection to the environment.

#### ***1.4. Carbon and its allotropes***

Carbon is the most important element found on Earth, and it yields the basis of all living organisms. Organic carbon it's found in animals and plants as a constituent of proteins, amino acids, lipids and nucleic acids. After millions of years, it can be transformed into fossil fuels (Loos 2015). Inorganic carbon is present in diamonds, pencils, lubricants and polymeric materials (Loos 2015). Carbon has wide technological application, varying from biomedicine to synthetic materials, due to its capacity to bind to itself and to almost all the elements (Hirsch 2010).

Basic carbon forms exist in two natural allotropes: diamond and graphite (Hirsch 2010). Diamond has a cubic and hexagonal configuration and represents  $sp^3$ -hybridized carbon atoms (Loos 2015; Hirsch 2010). Graphite is a very stable thermodynamic material and its structure is formed of sheets of atoms with an hexagonal arrangement, known as graphene (Loos 2015) (Figure 1).

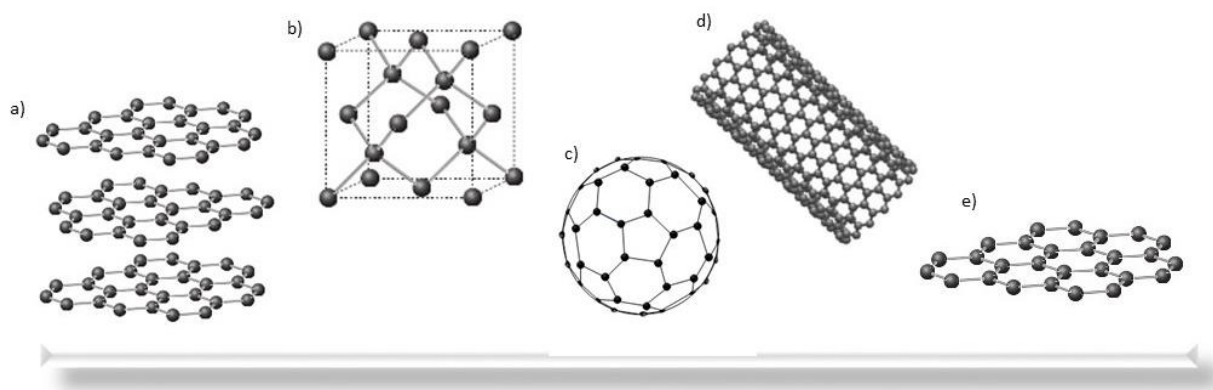


Fig. 1. Schematic representation of carbon allotropes: a) graphite, b) diamond, c) fullerene, d) carbon nanotubes, e) graphene (adapted from Loos 2015).

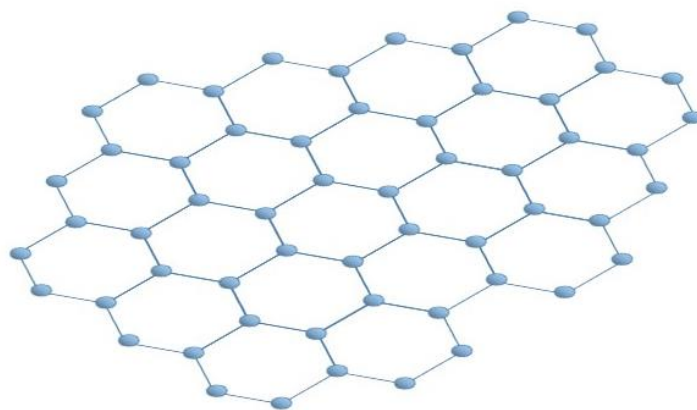
In 1985, Kroto et al. discovered a new allotrope, called fullerene ( $C_{60}$ ), which is composed of a hollow cluster with 60 carbon atoms (or more), disposed as hexagons and pentagons (Loos 2015). Because of this discovery, Robert F. Curl Jr., Sir Harold Kroto and Richard E. Smalley were awarded with the 1996 Nobel Prize in Chemistry. For their application, fullerenes are chemically functionalized, because this confers them exclusive properties, such as increased solubility in any solvent (Hirsch 2010).

A few years later, Iijima (1991) synthesized another allotrope, the carbon nanotubes (CNTs), consisting of needle-like tubes. CNTs can be single-walled or multi-walled and usually they have diameters in the order of one or two nanometers, but can be a few millimeters in length (Hirsch 2010). CNTs are chemically resistant, the electrical conductivity can be controlled and they can produce refined molecular structures (Takamura 2009).

Finally, in 2004, Andre Geim and Konstantin Novoselov were able to prepare single-graphene layers by simple mechanical exfoliation of graphite (Novoselov et al. 2004). The isolation of this major carbon allotrope awarded them the 2010 Nobel Prize in Physics.

### 1.4.1. Graphene

When graphene was described, it immediately proved to be a compound of great potential due to all their physicochemical properties (Novoselov et al. 2004). Graphene consists of a monolayer of carbon atoms rearranged in a bi-dimensional honeycomb-like structure (Figure 2).



**Fig. 2.** Graphene has a bi-dimensional honeycomb-like structure.

Until the discovery of graphene, it was assumed that bi-dimensional compounds were thermodynamically unstable unless they were part of a 3D structure (Geim et al. 2007). The isolation of graphene showed that the crystals had a high quality and were stable, showing that it would be possible to have reliable 2D materials (Novoselov et al. 2004; Geim et al. 2007).

Mechanical exfoliation (repeated peeling) was the technique first used to isolate graphene films, resulting in graphene films of about 10  $\mu\text{m}$  in size (Novoselov et al. 2004). It is also possible to isolate graphene through chemical exfoliation, interspersing graphite with atoms and molecules so that their sheets are separated layer by layer. Furthermore, graphene can be obtained by Molecular Beam Epitaxy (MBE), which offers viable ways to produce graphene for electronic applications (Geim et al. 2007). However, even with all the existing techniques and all efforts to improve them, the mechanical exfoliation is still the technique that produces graphene with higher quality (Singh et al. 2011).

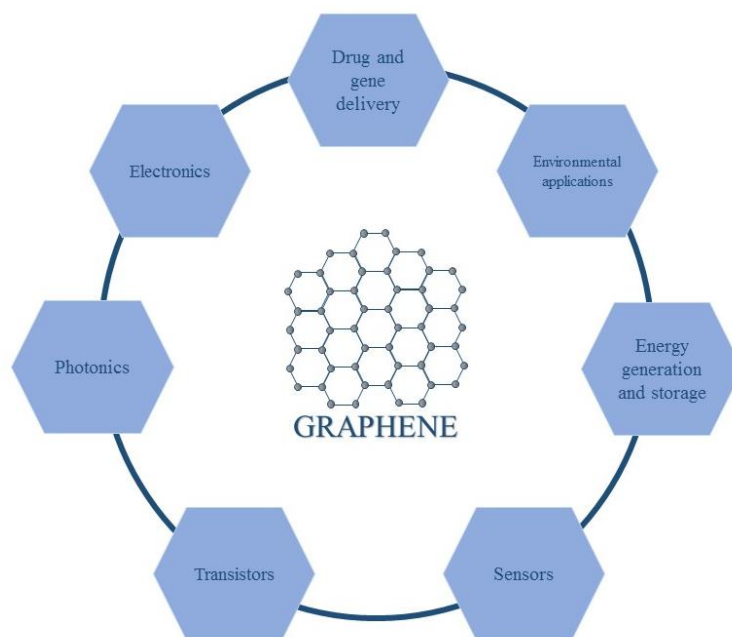
An extensive work is being done to understand all the potentials of graphene. It has special interest due to its exceptional properties. The optical properties of graphene are related to its unique electronic structure, showing an absorption of 2.3% of incident light, comprising

a wide wavelength range (Nair et al. 2008). Besides this, graphene has photoluminescence that can be used in cellular imaging (Sun et al. 2008). Once again, the bi-dimensional honeycomb-like structure, only a single-atom-thick provides exclusive electronic properties to graphene (Geim et al. 2007). This structure results in the Dirac point, where two bands combine (Chang et al. 2013). As electrons propagate through the carbon grid, they lose their mass completely, causing them to behave as relativistic particles (Chang et al. 2013). The mechanic properties of graphene are also extraordinary, following carbon nanotubes, it is the element with highest elastic modulus and strength (Singh et al. 2011). The perfect graphene single-atom-layer is flawless, causing it to have an intrinsic resistance similar to that of graphite (Singh et al. 2011). In terms of thermal properties, the single-atom-layer of graphene presents the highest thermal conductivity at room temperature (Balandin et al. 2008).

#### **1.4.1.1. Applications**

Because of its unique characteristics, graphene is an impressive material (Geim et al. 2007). Its physical and chemical properties increase the interest of scientists around the world and in different areas.

The physical properties of graphene make it an excellent material for applications on electronics and its high carrier mobility has gained greater attention (Avouris et al. 2012). Graphene has been applied in components such as transparent conductive coatings like touch-screen displays, e-paper and organic-emitting diodes (OLEDs) (Novoselov et al. 2012). The electronic products demand for a high transmittance and a low sheet resistance that are present in graphene. Due to his high conductivity, graphene has also been used to develop ultra-thin transistors (Mattevi et al. 2012). Supercapacitors (Liu et al. 2010) are also an area of interest since they are energy store devices that provide high currents. This supercapacitors can be applied for example to power electric cars (Randviir et al. 2014). Another potential application of graphene are photovoltaic cells, or solar cells (Wang et al. 2013). Along with great electronic properties, graphene has particular optical features over an ample wavelength range. Graphene photodetectors are widely studied (Mueller et al. 2010; Xia et al. 2009) and they can be used in a broad range, from ultraviolet to infrared wavelengths (Novoselov et al. 2012).



**Fig. 3.** Main fields of application of graphene.

As a result of an unique bi-dimensional structure, graphene becomes very sensitive to the environment and because of that it is used for sensor applications (Novoselov et al. 2012). Studies have demonstrated the ability to detect a diversity of molecules like gases (Dan et al. 2009; Robinson et al. 2008) and biomolecules (Ohno et al. 2009; Mohanty et al. 2008). Graphene's features make it a potential compound for bio-applications. Single-layered graphene has been the subject of investigation for its possible application on drug and gene delivery. Owing to the large surface area and easy surface functionalization, graphene and its derivate have been considered as a possible choice for multimodal imaging allying cancer photothermal and chemo- and/ or photodynamic therapies (Yang et al. 2015). The first demonstration of the use of graphene oxide for drug delivery was made by Liu and its group in 2008 (Liu et al. 2008). They found that nano-graphene oxide functionalized with polyethylene glycol (PEG), was physiologically stable and that there was no obvious toxicity for the concentrations tested, indicating that nano-graphene oxide alone was not toxic. Nowadays the applications of graphene have extended to many studies in drug delivery, bio-imaging and biosensors (Feng et al. 2013; He et al. 2010; Jung et al. 2010). Furthermore, graphene has also potential to be used in the field of regenerative medicine and tissue engineering (Nayak et al. 2011). Park (2011) and co-workers found that graphene could be used to stimulate the differentiation of human neural stem cells (hNSCs)

in neurons instead of glia. Therefore these studies support the use of graphene as a scaffold for stimulating NSC adhesion and differentiation, and also for neural prosthetics in neural regenerative medicine.

In virtue of its one-atom-thick layer, conductivity and strength, graphene is an excellent support for imaging biomolecules in transmission electron microscopy (TEM) (Nair et al. 1993). Graphene has a very organized and stable structure and these features make it a good support layer.

Graphene-based nanomaterials have also been capturing the attention for environmental purposes (Perreault et al. 2015). These materials have been used as electrodes materials for contaminant monitoring or removal (Zhao et al. 2011; Yan et al. 2014), sorbent or photocatalytic materials for decontamination (Gao et al. 2012) and as new materials for water treatment and desalination membranes (Ning et al. 2012).

In addition to all these properties, graphene has also been referred as the most recent antibacterial agent (Liu et al. 2011). However, its antibacterial effect is still controversial (Hegab et al. 2016), lacking more studies and analysis in this area.

#### **1.4.1.2. Graphene-family nanomaterials**

The intense research and the growing development of applications for graphene have led to the production of graphene derivatives, the so-called Graphene-Family Nanomaterials (GFNs). Graphene-derived materials may differ in surface chemistry, layer number, composition, lateral dimension, purity and defect density (Sanchez et al. 2012).

- **Bilayer graphene, trilayer graphene:** it consists in a stack of 2 or 3 graphene layers that are well-defined (Bianco et al. 2013).
- **Few-layer graphene (FLG):** it can be defined as stacked graphene sheets, usually 2-5 layers (Bianco et al. 2013).
- **Multi-layer graphene:** like the above, it's a bi-dimensional structure with 2 to 10 graphene monolayers stacked and well-visible (Bianco et al. 2013).
- **Ultrathin Graphite:** it is a material with more than 10 graphene sheets stacked (3-5nm) but less than 100 nm. It is considered a nanomaterial since it has at least one dimension in the nanoscale (1-100nm) (Sanchez et al. 2012).

- **Exfoliated Graphite:** this definition is based on the manufacturing process. It's a material produced from a partial exfoliation of graphite, resulting into thin parcels with the original 3D stacking of graphite (Bianco et al. 2013).
- **Graphene Nanosheet (GN):** it is a single-layer of graphene in suspension or adhered to a substrate (Bianco et al. 2013).
- **Graphene Microsheet:** it is a material similar to that previously described, but its dimensions range from 100nm to 100µm (Bianco et al. 2013).
- **Graphene nanoribbon:** it is a single-atom-thick band of carbon atoms where the longer lateral dimension exceed the width by at least one order of magnitude (Bianco et al. 2013).
- **Graphene Quantum dots (GQDs):** it is a term used to describe few-layered graphene nanosheets in studies about photoluminescence. Its lateral dimensions are <10nm (Bianco et al. 2013).
- **Graphene Oxide (GO):** it is a graphene monolayer that has a functionalized surface, with an elevated content in oxygen (Sanchez et al. 2012). This material is produced by oxidation and exfoliation of graphene (Bianco et al. 2013).
- **Graphite Oxide:** it is a solid material resulting from the oxidation of graphite. This material can be used to produced graphene oxide monolayers (Bianco et al. 2013).
- **Reduced Graphene Oxide (rGO):** it is formed by reducing the oxygen content of graphene oxide (Bianco et al. 2013). This process is applied in order to build up the electrical conductivity and alter other GO properties (Sanchez et al. 2012).
- **Nano-GO:** normally describes graphene oxide with small lateral dimensions (20-100nm). Owing to its small size, it can be used for biological applications: it is a stable material and it easily enters the cell (Sanchez et al. 2012).

#### 1.4.1.3. Graphene's Toxicology

Nanomaterials have been widely used in consumer and industrial products and this situation brings up many questions regarding their interaction with biological systems and consequently, the associated risks. Because of that, a lot of studies have been made, trying to elucidate us about the possible hazards of these materials. One important thing is the fact that the toxicity experiments are prepared and standardized for studies in chemical

products (Kroll et al. 2009). The nanoparticles have a lot of exclusive physicochemical features that may cause some conflicts to the classical toxicity assays (Dhawan et al. 2010). As described above GFNs are emergent materials with applications in many areas. Because of their extensively manufacturing in the last years, the probability of human exposure by inhalation, ingestion, dermal penetration and injection or implantation has dramatically increased (Xia et al. 2009).

Some studies have reported cytotoxicity of GFNs in many bacteria, mammalian cells and animal models. Graphene oxide and reduced-graphene oxide are the most studied graphene's derivatives because of their high solubility/dispersibility/stability in water, when compare to other GFNs (Guo et al. 2014).

Hu and co-workers (2010) showed the antibacterial power of graphene-based paper and that it can be produced with high inhibition ability to bacteria growth. Neural Phaeochromocytoma-derived PC12 cells were used to compare toxicity between graphene and single-wall carbon nanotubes (SWCNTs) (Zhang et al. 2010). In this study was demonstrated that both graphene and SWCNTs induced cytotoxic effects and the effects were dependent of the concentration and the shape of the graphitic material. Exposure to SWCNTs greatly increased the LDH release when compared to graphene, while exposure to graphene induced the generation of ROS and caspase-3 activation indicating apoptosis induction (Zhang et al. 2010). Jaworski et al. (2013) evaluated the toxicity of graphene platelets (GP) on U87 and U118 glioma cells, by analyzing the effect of this nanomaterial on the morphology, viability, membrane integrity and mechanisms of cell death. GP proved to be toxic for glioma cells, but apoptosis was only triggered in U118 cell line. Nevertheless, due to the large surface area of the GP (between 450nm and 1.5µm – not at nanoscale) they were not observed inside the cells. Kunming mice were used to study *in vivo* the toxicity of GO with doses of 0.1, 0.25 and 0.4mg/kg for 1 day, 7 days and 30 days, respectively (Wang et al. 2011). At small concentration (0.1 and 0.25 mg/kg) doses, the GO exposed intravenously, showed no toxicity, however a higher dose (0.4 mg/kg) caused chronic toxicity. Mortality was observed due to obstruction of the major airways as consequence of GO accumulation. Tissue analysis showed lung inflammation and formation of epithelial granulomas. The results also demonstrated accumulation of GO in liver.



Inhalation is one of the major means of exposure to graphene nanomaterials. The inhalation toxicity of graphene nanoplatelets (GNP) and carbon black was analyzed in C57BL/6 mice (Schinwald et al. 2012). Mice were treated by pharyngeal aspiration, intrapleural injection, succeeded by lavaging the lung and pleural space. After exposure with GNP, it was observed an increase in pro-inflammatory cytokines, in contrast with carbon black and control.

The characterization of nanomaterial to be tested is of extreme importance and the size is the most essential parameter; it is crucial for understand the interactions with biological systems (Dhawan et al. 2010). Among the several techniques used to evaluate the nanoparticles sizes, the most frequent are Brunauer-Emmett-Teller (BET), transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS) and atomic force microscopy (AFM) (Dhawan et al. 2010).

## **2. Assay techniques and biomarkers to access nanoparticle toxicity**

For correct assessment of the risks involved in the production and use of nanomaterials, it is essential to understand their effects on human health and quantify the levels of exposure (Oberdörster et al. 2007). The characteristics of nanoparticles can pose great challenges to the techniques used in toxicology and because of that it is important to have standards procedures, with controlled conditions (Kalantzi et al. 2014).

One of the most important steps for the study of nanoparticle cytotoxicity is the characterization of these nanoparticles. This information will allow to better understanding the interactions between the nanoparticles and the cells. Dynamic light scattering measures the hydrodynamic diameter of the NPs and it is possible to doing it in circumstances that simulate the exposure conditions. The AFM is an instrument that gives information about several properties of NPs: morphology, size, surface texture and roughness.

*In vitro* assays, using cell cultures, are widely used methods to evaluate the cytotoxicity of various compounds in cellular systems. The *in vitro* studies can include methods for measuring the metabolic activity of cells, using colorimetric assays. Other methods like LDH assay provides information about the integrity of the cell membrane. Bioluminescent methods are also used to assess the number of viable cells, with neutral red or TrypanBlue (Kalantzi et al. 2014). The study of cell cycle progression is also a very important method

in the evaluation of cytotoxicity of nanomaterials. These studies allow to understand if the exposure to nanomaterials may or may not interfere with the normal functioning of cell cycle. The production of reactive oxygen species (ROS) is one of the major mechanisms of nanoparticle toxicity and therefore it is essential to evaluate the amount of ROS produced by the cell upon nanomaterials exposure. Another essential parameter to consider in a toxicological assay is the NPs cellular uptake. This will allow the understanding of biological effects that NPs can cause. Cell death is also a parameter that can be analyzed in *in vitro* studies.

Toxicologists use *in vitro* studies always as a first approach instead of *in vivo*, because they provide faster results, they are less expensive and more convenient and besides that, there are no ethical issues to deal with. However, and because the methods are settled for chemicals, they must be applied with caution considering the physicochemical properties of NPs. It is also fundamental to consider that during the manufacturing process or managing in the laboratory, contaminants may be introduced and so alter the results (Dhawan et al. 2010).

The following sub-sections will describe some of these techniques in more detail:

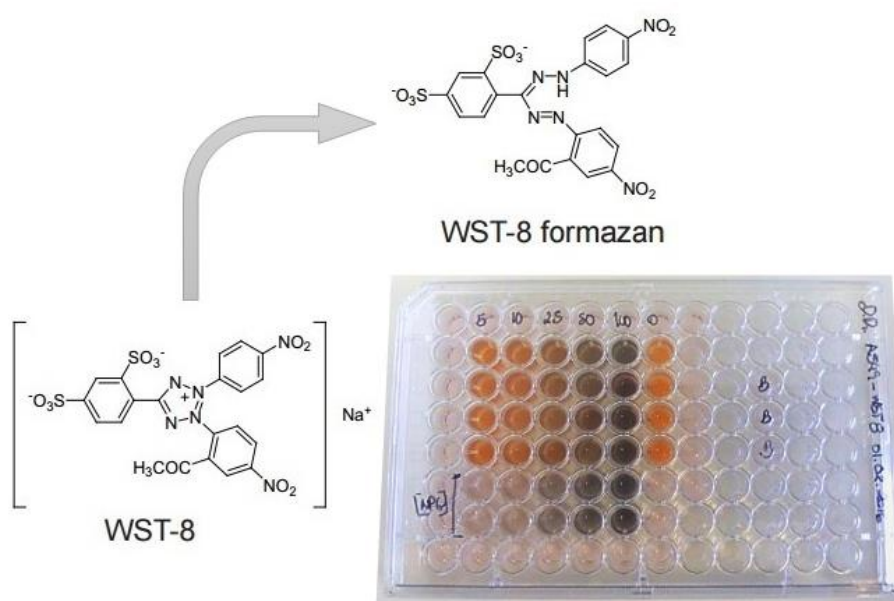
### **2.1. Cell viability – WST-8**

Cell viability studies are normally performed to understand if a given compound affects cell proliferation or show direct cytotoxic effects that eventually lead to cell death. There are a wide variety of experiments that can be performed to assess the viability of eukaryotic cells. One of the most common is based on tetrazolium compounds, as for instance MTT, MTS, XTT or WST series, that are used for evaluate the metabolic level of the cells (Riss et al. 2004). These assays require the cell incubation with the tetrazolium reagent that will be converted to a colored product that can be detected with a plate reader. The signal is directly proportional to the amount of viable cells in that population (Riss et al. 2004). If the cells are not viable, there is no reduction of the substrate and therefore, no signal is emitted. The signal intensity will depend on many factors such as the concentration, the incubation time, the amount of viable cells and their metabolic activity (Riss et al. 2004).

The MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide] assay is one of the most widely used techniques to ascertain cell viability. MTT is reduced mostly

by NADH of metabolically active cells, that convert MTT into an intense purple colored formazan compound, which is water insoluble (Riss et al. 2004), therefore, it has to be solubilized before the absorbance measurement (Tominaga et al. 1999). This further step in the protocol makes it more time-consuming, which can represent a disadvantage. The other tetrazolium reagents, such as MTS, XTT and WST series are soluble in cell culture medium, which makes the assay procedure much more simple (Riss et al. 2004).

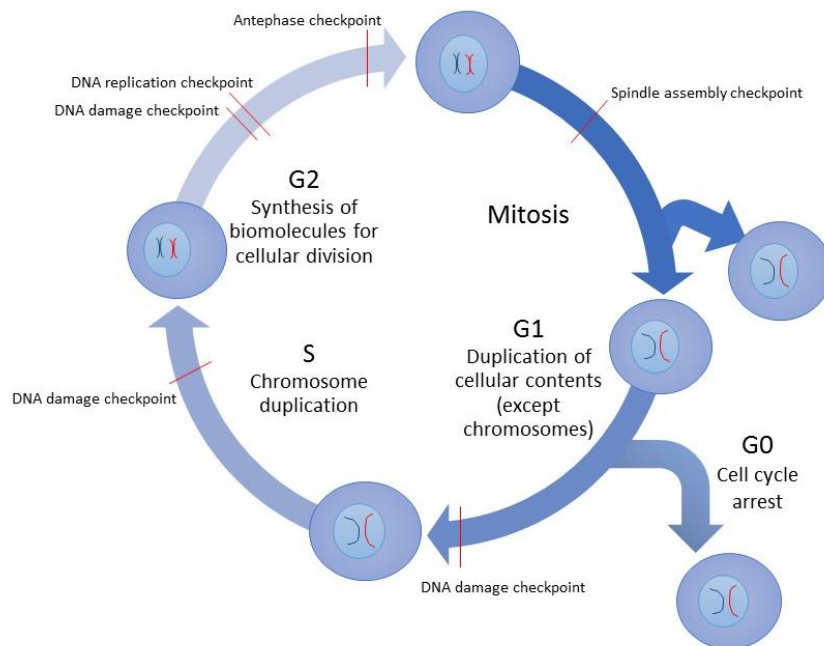
In this study it was used the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], which is a very responsive, stable salt and it is easy to synthesize (Ishiyama et al. 1997) (Figure 4). As described by Tominaga et al. (1999), the WST-8 has a higher sensitivity when compare with conventional tetrazolium salts.



**Fig. 4.** The WST-8 is a highly water-soluble tetrazolium salt that is reduced to an orange-coloured formazan dye in viable cells. The figure shows a 96-well plate used in this study.

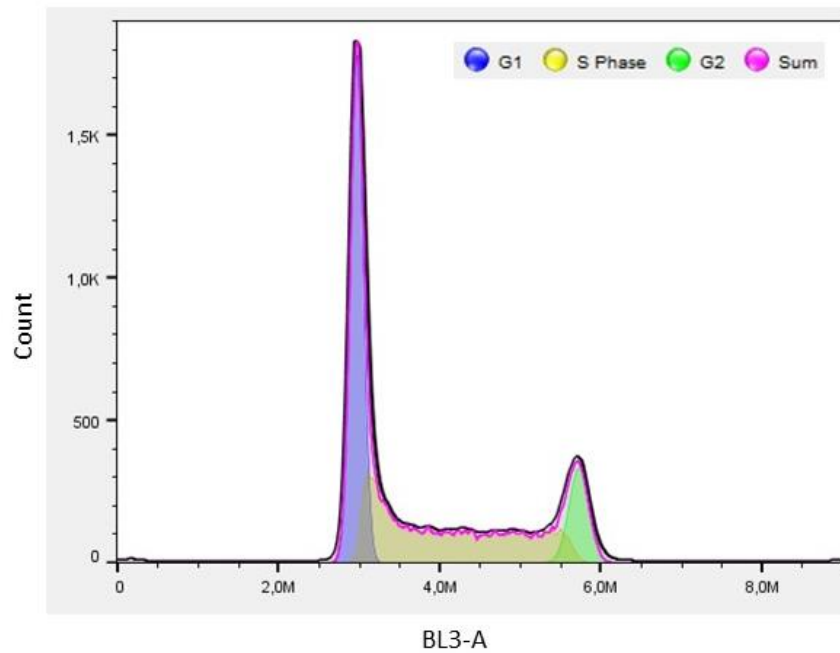
## 2.2. Cell cycle analysis

The cell cycle is a succession of events in a cell that lead to duplication of all of its elements and to its division in two daughter cells (Dubitzky et al. 2013). The cell cycle is conventionally divided into five phases: G<sub>0</sub> (Gap), G<sub>1</sub> (First Gap), S Phase (Synthesis Phase), G<sub>2</sub> (Second Gap) and M Phase (Mitosis) (Figure 5). G<sub>1</sub> is characterized by an intensive biosynthetic activity of proteins, enzymes and RNA followed by a cell growth; S Phase is the synthesis phase during which the DNA is synthesized and replicated; G<sub>2</sub> is a period of synthesis of biomolecules necessary to the cellular division that is followed by the mitotic phase (M Phase) (Jayat et al. 1993). Non-proliferating cells enter a quiescent state at G<sub>1</sub> that prevents further divisions – the G<sub>0</sub> Phase. During the cell cycle, it is required a regulation, to guarantee that all the steps follow the right direction. The cell does this regulation through the cell cycle checkpoints. There are two important checkpoints: the first ensures the right DNA replication (at the G<sub>1</sub> to S checkpoint), and the second ensures the successful completion of the early mitoses (at G<sub>2</sub> to M checkpoint) (Dubitzky et al. 2013). There's also a third checkpoint at the M phase and it guarantees that each daughter cell receives a complete copy of the genome (Ciliberto et al. 2009). These checkpoints work through a machinery of complexes of cyclins and cyclin-dependent kinases (Dubitzky et al. 2013).



**Fig. 5.** Schematic representation of cell cycle and its checkpoints for DNA damage control (adapted from Chin et al. 2010).

Flow cytometry is used to analyze various cellular components, organelles and functions, and it is widely used to study the cell cycle by taking DNA content into account (Jayat et al. 1993). The cell cycle analysis allow us to estimate the portion of cells that are in each phase through analytical processes (Dubitzky et al. 2013) (Figure 6).



**Fig. 6.** Cell cycle profile of A549 by flow cytometry.

These studies are carried out with specific DNA dyes and it is accepted that the fluorescence emitted is proportional to DNA content in the cells (Kerker et al. 1982). If the cells are in the  $G_1$  phase, the DNA content will be 1x and it will form a peak in the DNA distribution; if cells are in  $G_2$  phase, the DNA content will be 2x and it will form a peak in the DNA distribution twice the peak of  $G_1$ . The cells in the S phase will have an intermediate DNA distribution (Gray et al. 1986).

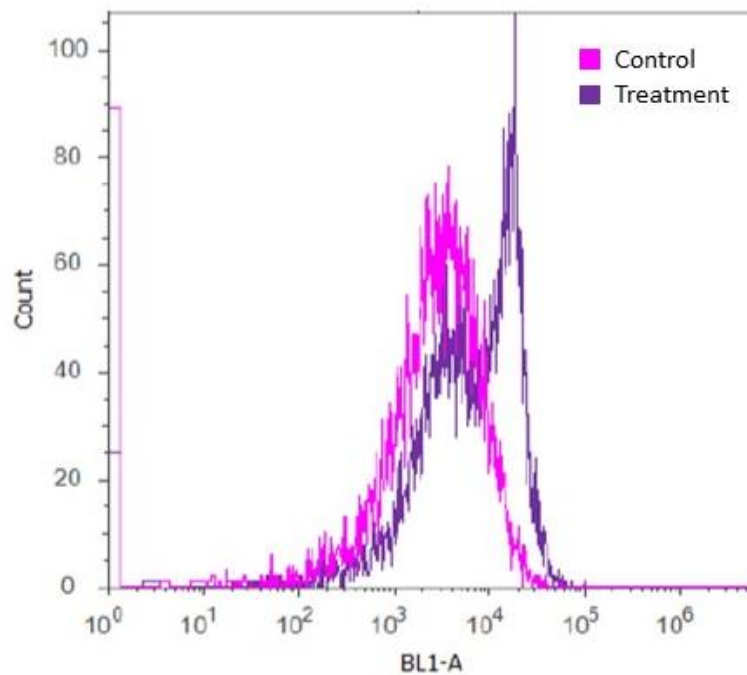
Flow cytometry gives us information by analyzing cell by cell and so it is possible to do a targeted study, with quantitative information, quick and accurate (Jayat et al. 1993).

### **2.3. Intracellular ROS detection**

Reactive Oxygen Species (ROS) are small, short-lived and highly reactive molecules, formed by incomplete one-electron reduction of oxygen (Scherz-Shouval et al. 2007). This group contains oxygen anions, free radicals, such as superoxide and hydroxyl radical, and peroxides, such as hydrogen peroxide ( $H_2O_2$ ) (Scherz-Shouval et al. 2007). ROS are continuously produced under normal conditions as a consequence of aerobic respiration (Eruslanov et al. 2010). They are also involved in enzymatic reactions, mitochondrial electron transport, signal transduction, activation of nuclear transcription factors, gene expression and the antimicrobial action of neutrophils and macrophages (Bayr et al. 2005). However, the generation of reactive oxygen species is an important mechanism of nanotoxicity, inducing oxidative stress in cells (Fu et al. 2014).

Free radical processes are involved in cellular physiology and pathology and therefore it is essential to use a method to quantify the production of ROS in living cells (Jakubowski 2000). The most common techniques use cell permeable fluorescent and chemiluminescent probes. 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used probes for measuring the redox state of a cell. DCFH-DA is cell permeable and a non-fluorescent precursor of DCF (2'-7'-dichlorofluorescein) that is used as an intracellular probe (Eruslanov et al. 2010). This probe is very sensitive to changes in the redox state of the cell and can be used to monitor ROS over time (Eruslanov et al. 2010).

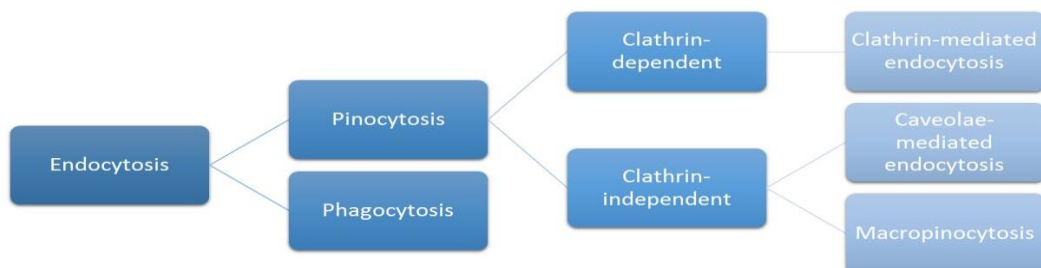
After adding DCFH-DA to the cells, it diffuses across the cell membrane and it is hydrolyzed by intracellular esterases, to release DCFH, which reacts with oxidizing species, forming an extremely fluorescent compound, DCF (Rota et al. 1999), that can be detected either by fluorescence microscopy or flow cytometry (Figure 7). The redox state of the sample can be observed by the intensity of the fluorescence emitted (Eruslanov et al. 2010).



**Fig. 7.** Detection of intracellular ROS by flow cytometry.

#### ***2.4. Nanoparticle cell Uptake***

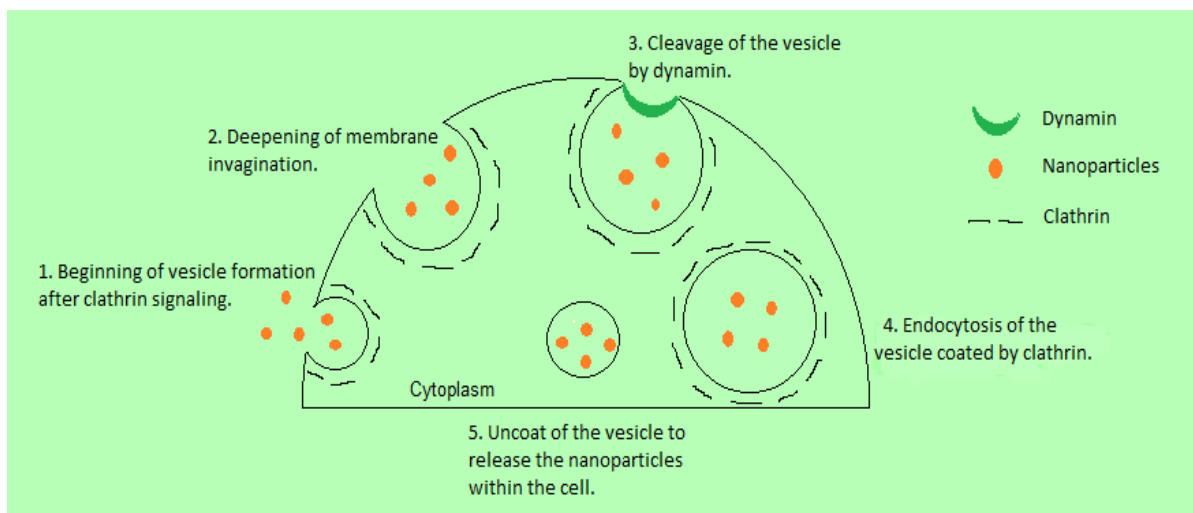
The nanoparticles have been the subject of several studies, due to their potential application in the medical field. One of the parameters to be considered is the interaction of these NPs with the cells and how these interactions can influence their uptake. The physical properties of NPs have a fundamental role in their entry into the cell, as well as all the biological environment and the characteristics of the cell membrane itself (Adjei et al. 2014). There are numerous receptor-mediated pathways for the internalization of substances that may occur simultaneously (Figure 8).



**Fig. 8.** Mechanisms of endocytosis for cell uptake.

One of the most common mechanisms for uptake is phagocytosis and it is present in various types of cells (Aderem et al. 1999). In mammals, phagocytosis act as a response of the immune system, engulfing all the unwanted particles in the organism (Silverstein 1995) (Figure 10). Phagocytosis works with the help of vesicles known as phagosomes, which have sizes greater than 250 nm (Rabinovitch 1995). The interaction of the cell receptors with unfamiliar particles can lead to the activation of this process; however, it can also be activated by means of opsonins that can attach the foreign particle and facilitates phagocytosis (Yameen et al. 2014).

Pinocytosis is another mechanism used by cells for the uptake of substances and can be further divided in clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis (Yameen et al. 2014) (Figure 10).

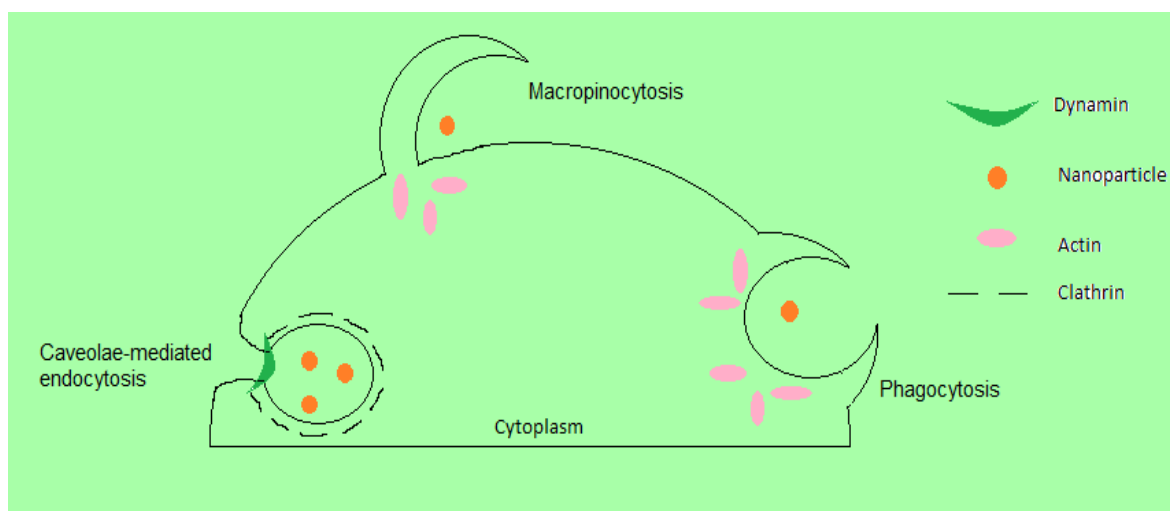


**Fig. 9.** Illustration of the mechanism of clathrin-mediated endocytosis (adapted from Murugan et al. 2015).

Clathrin-mediated endocytosis involves the formation of a clathrin coat around a vesicle (Murugan et al. 2015). The pathway is activated by the signaling of substances and then various proteins start to work on the formation of the vesicle. Clathrin is recruited from the cytosol to form the coat and finally, the vesicle detaches from the cellular membrane and goes to the interior of the cell (Figure 9). Once there, it releases the endocytosed substances (Xiang et al. 2012).



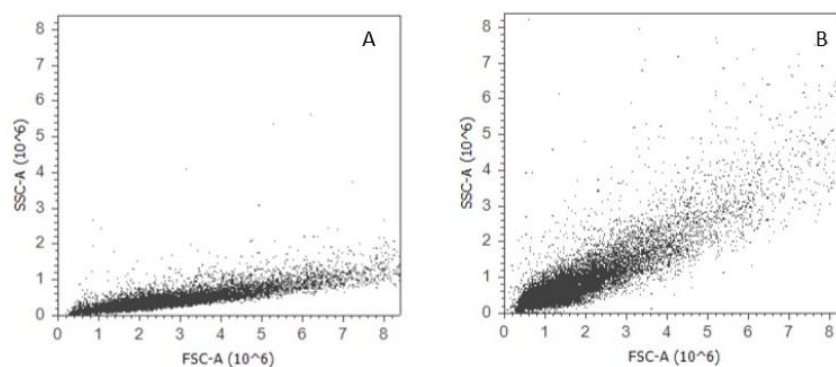
Caveolae-mediated endocytosis is a mechanism that initially forms a flask-like shape structure with the help of caveolins that bind with the cholesterol present in the cellular membrane (Kumari et al. 2010). The actin present in the cellular membrane is reorganized and dynamin is recruited from the cytosol to form the caveolae structure (Pelkmans et al. 2002) (Figure 10). The internalization of the vesicle is similar to the clathrin-mediated endocytosis.



**Fig. 10.** Illustration of caveolae-mediated endocytosis, macropinocytosis and phagocytosis (adapted from Murugan et al. 2015).

Macropinocytosis is an independent process that allows the internalization of large volumes of extracellular fluids (Kerr et al. 2009). The actin present in the cellular membrane is polymerized, allowing the formation of protrusions that encapsulates the fluids (Kumari et al. 2010) (Figure 10). These protrusions fuse with the membrane forming macropinosomes (Kerr et al. 2009). The fate of the macropinosomes appears to be different according to the cell type that they are formed; they may enter the endosomal/lysosomal pathway or they can remain isolated within the cell (Kerr et al. 2009).

The uptake of NPs at the cellular level can be assayed by flow cytometry. This technique combines two properties of scattered light laser: the forward-scatter (FS) and the side-scatter (SS) (Figure 11).



**Fig. 11.** Analysis of incorporation of nanoparticles by the flow cytometric light scatter parameter. A) Control, B) Treatment.

The intensity emitted by these two parameters is proportional to the size of cells (FS) and the intracellular density (SS) (Suzuki et al. 2007). In the experiment conducted by Suzuki and co-workers (2007), they concluded that cells which assimilated a larger number of NPs showed a higher SS.

## 2.5. *Apoptosis/ Necrosis*

Apoptosis consists of a mechanism of programmed cell death and it is essential during development and in the maintenance of homeostasis (Hollville et al. 2016). When a cell enters apoptosis there are many morphological modifications: cell shrinkage, bebbing of plasma membrane, nuclear chromatin condensation and fragmentation, with maintenance of organellar integrity (Mattson et al. 2012). Biochemical events also occur like the upregulation of proapoptotic proteins such as Bax and Par-4; the release of cytochrome c; activation of effector caspases; externalization of phosphatidylserine (PS), which serves as a signal for apoptosis recognition (Mattson et al. 2012).

Normally, necrosis occurs as the result of severe insults, such as trauma, cellular energy failure or toxicity (Mattson et al. 2012). In the course of necrosis, the changes that happen are different from apoptosis. The cell swells, the organelles lose their integrity and the nuclear membrane becomes disrupted; this process is also independent of proapoptotic proteins (Mattson et al. 2012).

Apoptosis is usually an individual process, there is no release of cellular contents and the dying cell is engulfed by macrophages; on the other hand, during necrosis, the cell lyses and it releases its contents that can damage the surrounding cells (Mattson et al. 2012).

Phosphatidylserine is a plasma membrane phospholipid concentrated on the inside (Fadok et al. 1998). When cells enter apoptosis, the plasma membrane lose its phospholipid asymmetry and expose PS on the surface of the membrane, which works as a marker for apoptosis (Fadok et al. 1992). Flow cytometry assays use the fluorochrome-labelled Annexin-V that binds to PS in a calcium-dependent manner (Fadok et al. 1998; Raynal et al. 1994). A vital dye such as propidium iodide (PI) can be combined with Annexin-V, to distinguish between live, apoptotic and necrotic cells, according to differences in membrane permeability (Vermes et al. 1995; Vermes et al. 2000). PI is not permeable to membranes and so, live and apoptotic cells are not stained (Vermes et al. 1995; Vermes et al. 2000; Darzynkiewicz et al. 1992). During late apoptosis or necrosis, the permeability of plasma and nuclear membranes changes and PI enters the cell and intercalates into DNA allowing the identification of the nucleus by emitting its characteristic red fluorescence (Vermes et al. 1995; Vermes et al. 2000; Darzynkiewicz et al. 1992).

### **3. A549 Cell line**

A549 cell line comes from a human lung adenocarcinoma. It was first isolated in 1973 by Giard et al. (1973) from a cancerous lung tissue culture of a 58 years old Caucasian man. During its analysis, these cells showed multilamellar cytoplasmic inclusion bodies, a characteristic of Type II alveolar epithelial cells of the lung (Lieber et al. 1976). These cells are used as a research model on epithelial lung structures and in studies relating to the development of lung cancer (Jiang et al. 2010). In addition, these cells are one of the most applied model for toxicological studies in air pollution (Líbalová et al. 2014).

#### **4. Aims**

The lung is the major route of entry of the nanoparticles in the human organism and so, it is important to understand the effects of the graphene nanoparticles on their cells.

The general aim of the present dissertation was to evaluate the cytotoxic potential of graphene nanoplatelets on lung cells.

The specific aims of this work were:

- Characterization of the graphene nanoplatelets in terms of their physicochemical properties;
- Evaluate the effects of graphene nanoplatelets on the cell viability on the human lung adenocarcinoma cell line (A549);
- Evaluate the effects of graphene nanoplatelets on cell cycle progression;
- Elucidate the type of eventual cell death induced by graphene nanoplatelets;
- Evaluate the putative ROS induction in A549 cells by graphene nanoplatelets;
- Assess the nanoparticle uptake by flow cytometry.

## **5. Material and Methods**

### **5.1. *Graphene nanoplatelets***

Graphene nanoplatelets (GNPs) were purchased from Sigma-Aldrich® dispersed in H<sub>2</sub>O (1mg/ml). According to the manufacturer, there is a presence of a few-layer graphene flakes with an average of 5-7 atomic layers.

### **5.2. *Characterization of Graphene Nanoplatelets***

The hydrodynamic diameter of GNPs was measured by Dynamic Light Scattering – DLS – and the zeta potential was assessed by electrophoretic mobility, both measurements using a ZetaSizer Nano ZS (Malvern Instruments, UK). This method allows us to measure the hydrodynamic diameter (effective diameter of a particle in a liquid environment) of the particles in suspension in the cell culture medium and Milli-Q water. The concentration used was 10µg/ml and the reads were done at time zero, 2h, 4h and 24h.

### **5.3. *A549 cell line culture***

For this study, the human alveolar adenocarcinoma cell line A549 was used as a model. The A549 cell line was cultured in a 25 cm<sup>2</sup> flasks (Corning®) with 5ml of Kaighn's Modification of Ham's F-12 Medium (F-12K), supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco), 1% of penicillin-streptomycin (Gibco) and 1% Fungizone (Gibco). The cells were observed every day under the inverted microscope (Nikon®Eclipse TS100) to check if there was no contamination or any other problem with the culture and to understand its growth and confluence (% of cell coverage on the surface area of the culture vessel) state. Subculture was performed every 3-4 days, when cultures reached approximately 70-80% confluence. After removal of the culture medium, cells were washed with 5 ml PBS and incubated with 3 mL Trypsin-EDTA (0.25% trypsin, 1 mM EDTA) for 5 min, to cleave proteins that enable cell adherence to the flask and cell-cell adhesion. After cell detachment from the culture flask, trypsin was inactivated by adding 6 mL supplemented medium to the culture. Cells were harvested, counted using a hemocytometer and seeded in a new flask with complete culture medium. The cultures were maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere.

#### **5.4. *Graphene Nanoplatelets exposure***

Cells were seeded in multiwell plates and incubated for 24h at 37°C, 5% CO<sub>2</sub>, to adhere. After the incubation period, the medium was taken out and replaced for the same amount of the proper dilutions of GNPs. The time of exposure was normally 24h, with some assays with 4h of exposure.

#### **5.5. *Cell viability – Cytotoxicity Assay: WST-8***

Cell viability was measured using the WST – 8 assay (Sigma-Aldrich®) by following manufacturer instructions, with some modifications.

Briefly, in a 96 – well plate, one hundred µl of cells were seeded at the concentration of 7x10<sup>4</sup> cells/ ml and incubated for 24h, at 37°C, 5% CO<sub>2</sub> for cells adherence. After that, the culture medium was replaced by 100 µl of GNPs in growth medium at the following concentrations: 5, 10, 25, 50 and 100 µg/ml. Cells cultured in culture medium without GNPs were taken as the control. After 24h exposure, 10 µl of WST – 8 reagent were added to each well. Then, the plate was incubated for 1h at culture conditions and then the absorbance was measured at 450nm in a microplate reader (Biotek® - Gen5™ software). The cell viability was calculated using the equation, where A450 represents the absorbance of the sample read at 450nm:

$$\text{Relative \% viability} = \text{A450 sample} / \text{mean A450 control} \times 100$$

Some alterations were then performed to this basic protocol (Protocol I) in order to minimize the putative interference of the particles with the assay. The first alteration (Protocol II) included a washing step with PBS (50 µl, for 30 seconds) following the 24h exposure, and then complete culture medium with WST – 8 reagent was added to each well. The plate was then incubated and analyzed as described above. An additional experiment to test the adsorption of the culture medium by GNPs on the toxicity was performed according to the procedure described by Chang et al. (2011) (Protocol III). GNPs samples at 5, 10, 25, 50 and 100 µg/ml were incubated in culture medium (without cells) at 37°C, 5% CO<sub>2</sub>, for 24h. Then, the GNPs suspensions were centrifuged at 400 rpm for 5 min. The supernatants were then collected and were used to expose the cells as described above. After 24h incubation, cell viability was assessed with the WST-8 assay. For this assay it was used a background control for each concentration and a blank.

### **5.6. *Flow cytometric analysis of Cell Cycle***

Cell cycle distribution was analyzed according to the method previously described by Oliveira et al. (2014). Briefly, cells were seeded in 6 well plates each receiving 2 ml of suspended cells at the concentration of  $1 \times 10^5$  cells/ml. They were allowed 24 hours to adhere and grow in the incubator before being exposed to GNPs at 5, 50, 100  $\mu\text{g/ml}$  and the control. Each assay concentration consisted of three technical replicates. This was followed by an incubation time of 24h. Afterwards the media was removed and the cells were washed with PBS, trypsinized and resuspended in media. The suspensions were collected to microtubes and centrifuged at 300g for 5 min. The supernatant was discarded and the cells were washed in PBS and finally resuspended in 1 ml of cold ethanol 85% and then they were stored at  $-20^\circ\text{C}$  until analysis.

At the time of analysis, cells were centrifuged at 300g for 5 min at  $4^\circ\text{C}$ . Next, the supernatant was discarded and the cells were resuspended in 800  $\mu\text{l}$  of PBS. After this, the suspension was filtered through a 55  $\mu\text{m}$  nylon mesh to remove possible clusters. Subsequently, 50  $\mu\text{l}$  of RNase (Sigma-Aldrich®, USA) and 50  $\mu\text{l}$  of PI (Fluka, USA) were added. This step ensures RNA elimination by RNase and DNA staining by PI. This mixture was left in the tubes in the dark for 15 min and then read in the flow cytometer. To measure the fluorescence intensity, samples were analyzed with flow cytometer Coulter EPICS XL (Beckman Coulter Inc., Brea, CA, USA) and Attune® Acoustic Focusing Cytometer (Applied Biosystems). The number of events reached approximately 5000, for each sample. The data were analyzed by FlowJo software (FlowJo LLC, Ashland, OR, USA).

### **5.7. *Intracellular ROS detection assay***

For the measurement of the Reactive Oxygen Species (ROS), probe 2', 7'-dichlorofluorescein diacetate (DCFH<sub>2</sub>-DA) (Sigma-Aldrich®) was used.

Cells were seeded in 6-well plates at the concentration of  $1 \times 10^5$  cells/ml and were allowed to adhere for 24h. Then cells were exposed to GNPs at the concentration of 5, 50 and 100  $\mu\text{g/ml}$ , for 24h. After that, cells were washed with PBS and incubated for 30 min at  $37^\circ\text{C}$  in the dark with serum-free F12K medium with 10 $\mu\text{M}$  of DCFH<sub>2</sub>-DA. Cells were then washed with PBS, trypsinized and collected for analysis in the flow cytometer. The samples were analyzed with flow cytometer Coulter EPICS XL (Beckman Coulter Inc.,

Brea, CA, USA) and Attune® Acoustic Focusing Cytometer (Applied Biosystems). The data were analyzed by FlowJo software (FlowJo LLC, Ashland, OR, USA).

### **5.8. Uptake Potential of nanoparticles**

The uptake potential of GNPs by A549 was analyzed by flow cytometry as previously described by Bastos et al. (2016).

Briefly, cells were seeded in 6-well plates at  $1 \times 10^5$  cells/ml and allowed to adhere for 24h. Cells were then exposed to GNPs at 5  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$  and the control were exposed to culture medium for 4 and 24h in three different sets.

The first set was incubated for 4h at 37°C; the second set was incubated for 4h at 4°C (for endocytosis inhibition study) and the third was incubated for 24h at 37°C. After the incubation period, the cells were trypsinized, resuspended and collected for flow cytometry analysis. Cytometry was performed using the Attune® Acoustic Focusing Cytometer (Applied Biosystems). The data were analyzed by FlowJo software (FlowJo LLC, Ashland, OR, USA).

### **5.9. Apoptosis/ Necrosis assay – Annexin V/PI**

Apoptosis was measured by flow cytometry, using the FITC Annexin V Apoptosis Detection Kit, from BD Pharmingen™ (BD Pharmingen, San Diego, CA, USA), by double staining with Annexin V and PI. This double staining gives us the differentiation between early-stage apoptotic cells (Annexin V-FITC (+)/PI(-) cells), from late-stage apoptotic/necrotic cells (Annexin V-FITC (+)/PI (+)) and live cells (Annexin V-FITC (-)/PI (-)).

Cells were seeded in 6-well plates, at concentrations of  $1 \times 10^5$  cells/ml and incubated for 24h to allow adherence. Then, cells were exposed to the highest concentration of GNPs (in this case, 100  $\mu\text{g/ml}$ ). Plates were incubated for 24h at 37°C, 5% CO<sub>2</sub>.

After exposure, cells were trypsinized and transferred to microtubes. Subsequently, cells were washed twice in cold PBS and resuspended ( $1 \times 10^6$  cells) in *1x Binding Buffer Solution*. Following this, 100 $\mu\text{l}$  of each cell suspension was transferred to microtubes and incubated for 15 min in the dark with 5 $\mu\text{l}$  of PI and 5 $\mu\text{l}$  of Annexin V. Then, 400 $\mu\text{l}$  of *1x Binding Buffer Solution* were added, and the samples were analysed by flow cytometry.



For each sample 10000 cells were analysed and the percentages were calculated from the number of cells in each quadrant divided by the total number of cells. For the measurements it was used the Attune® Acoustic Focusing Cytometer (Applied Biosystems) and data were analyzed by FlowJo software (FlowJo LLC, Ashland, OR, USA).

#### ***5.10. Statistical Analysis***

At least 3 replicates, and two independent assays were performed for all experiments. Data analysis was performed using the software SigmaPlot version 12.0, by one-way ANOVA analysis of variance (One-way ANOVA) ( $p < 0.05$ ), followed by a Holm-Sidak test ( $p < 0.05$ ) to evaluate the significance of differences in the parameters.

## 6. Results

### 6.1. Graphene Nanoplatelets Characterization

To ascertain the hydrodynamic diameter distribution of the GNPs in cell culture medium, we used the DLS. The GNPs supplier did not provide information regarding to the size of the nanoplatelets. The hydrodynamic size measurements were performed for the concentration of 10  $\mu\text{g/mL}$  in cell culture medium for  $t=0\text{h}$ ,  $t=2\text{h}$ ,  $t=4\text{h}$  and  $t=24\text{h}$ . The results presented in Table 1 show that over time, the size of the nanoparticles remains constant, about  $268\pm 6\text{nm}$ . The zeta potential ( $\zeta$ ) of GNPs was measured in ultrapure water and in F-12K complete culture medium, with a concentration of  $10\mu\text{g/ml}$  of GNPs. As shown in Table 1, GNPs have a strong negative zeta potential in ultrapure water ( $\zeta$  -37.40 mV), but in culture medium this value was much less negative ( $\zeta$  -8.44 mV). The GNPs showed a PdI of 0.36 in water, indicating a moderate polydispersion, while in culture medium the PdI increased up to 0.49, indicating a broad polydispersion. This may demonstrate some degree of agglomeration.

**Table 1** – Z-average size (Z-Ave) (with respective polydispersity index PdI) and Zeta Potential ( $\zeta$ ) of GNPs dispersed in ultrapure water (Control) or in F-12K culture medium ( $10\mu\text{g/ml}$ ). The standards deviations were calculated from the 3 replicates.

Time (hours)	Z-Ave(nm)	PdI	$\zeta$ (mV)
<b>Water (control)</b>			
0h	$268.37\pm 8.96$	0.36	$-37.40\pm 0.72$
<b>F-12K</b>			
0h	$279.03\pm 2.90$	0.49	$-8.44\pm 2.39$
2h	$269.27\pm 3.61$	0.49	
4h	$261.90\pm 5.65$	0.48	
24h	$259.47\pm 9.60$	0.49	

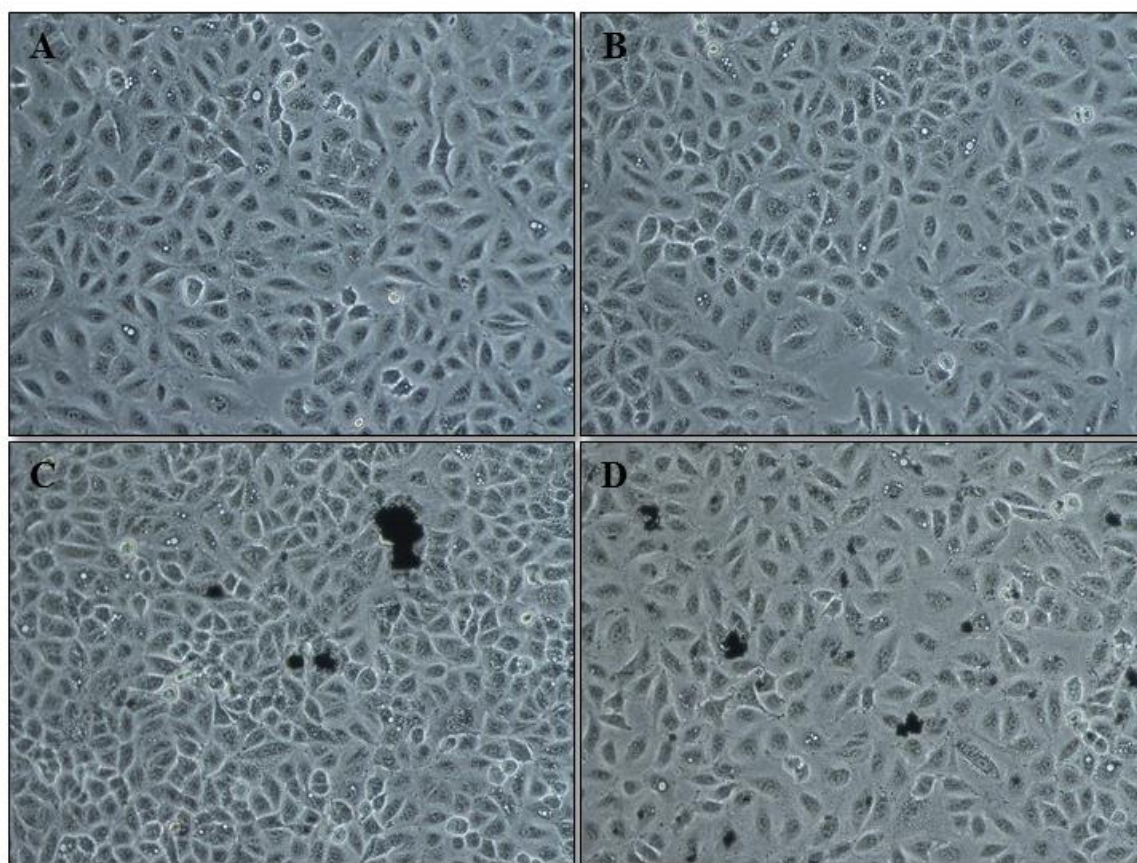
Table 2 represents the average size distribution of GNPs when suspended in water and complete culture medium. It is possible to observe that there are two peaks, one being substantially smaller than the other. This smaller peak is found in a higher percentage in the solutions. This results are in agreement with the PdI, showing a polydispersion of sizes.

**Table 2** – Average size distribution of GNPs suspended in ultrapure water (Control) and complete culture medium (F-12K). The average hydrodynamic size is represented as mean diameter (d.)  $\pm$  standard deviation (SD) in nm. The relative intensities of size peaks are indicated in %.

	Time (hrs)	Peak 1 d. $\pm$ SD (nm)	Peak 2 d. $\pm$ SD (nm)	Peak 3 d. $\pm$ SD (nm)	Peak 1 %	Peak 2 %	Peak 3 %
Water	0	250.2 $\pm$ 17.77	2397 $\pm$ 2728	-	94	6	-
F-12K	0	309.5 $\pm$ 5.378	4470 $\pm$ 2163	-	97.3	2.7	-
	2	310.5 $\pm$ 17.29	4423 $\pm$ 2169	8.145 $\pm$ 19.95	96.1	3.3	0.6
	4	293.7 $\pm$ 3.573	5374 $\pm$ 13.08	-	96.9	3.1	-
	24	284.0 $\pm$ 21.79	5261 $\pm$ 106	-	95.2	2.7	-

## 6.2. Confluence and Morphology

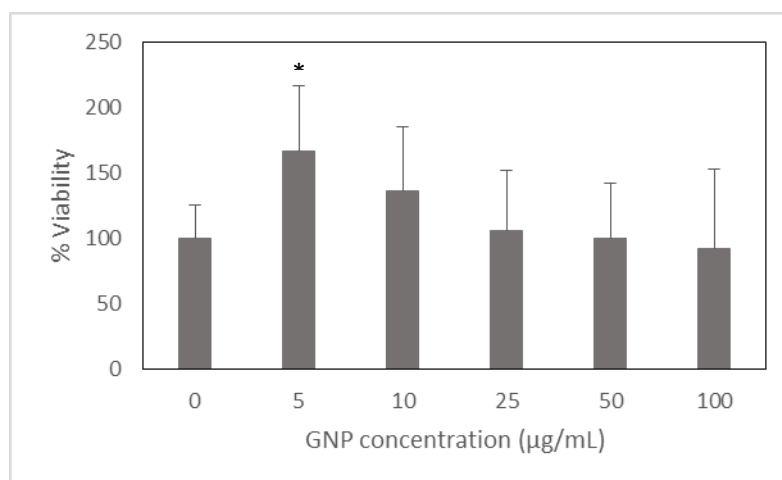
A549 cells grow in monolayers until confluence and they have an appearance of cuboidal and polygonal cells closely packed. These cells also grow rapidly and consistently as observed (they reach confluence in about 3 days). During the exposure period, cells were observed under the inverted microscope to verify confluence, morphology and possible contaminants. There was no visible loss of confluence, even at higher concentrations (Figure 12). However, it is possible to observe some agglomeration by the GNPs, being more evident at the higher concentration.



**Fig. 12** Light microscopy images of A549 cells exposed to GNP for 24h. A – Control; B - 5µg/ml; C - 50µg/ml; D - 100µg/ml. 40X.

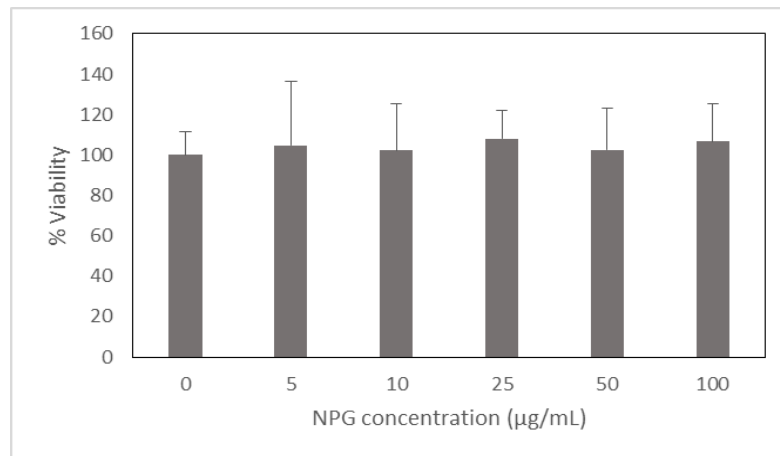
### 6.3. Cell viability – cytotoxicity assay: WST-8

To determine cell viability upon exposure to GNPs the WST-8 assay was used and cells were analysed after 24h exposure. The protocol was executed according to the information provided by Sigma-Aldrich®, with some modifications (Protocol I). To avoid interferences in readings from the reagent WST-8 and the medium a background control (without cells) was prepared, with typical absorbance between 0.1-0.2 O.D. To correct the values, we just subtracted the average absorbance of the background wells from that of the other wells. After 24h exposure to the GNPs it was observed a significant increase on cell viability at the lowest concentration (5 µg/ml) ( $p < 0.001$ ), with a slight decrease on the other concentrations, however without a significant statistical difference (Figure 13).



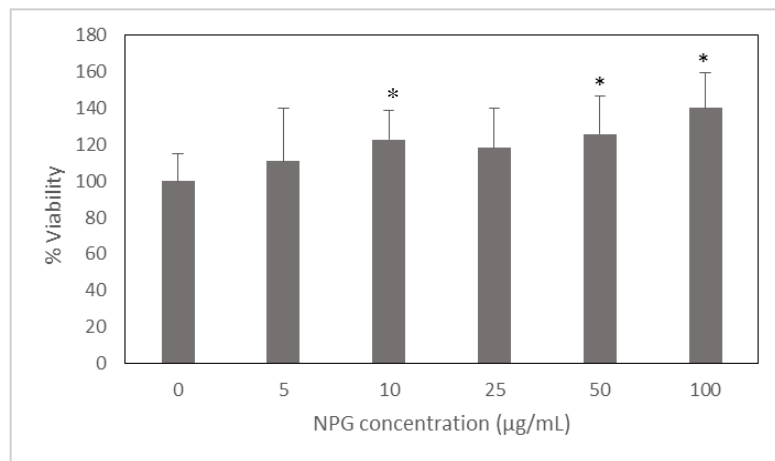
**Fig. 13.** Effects of GNP in A549 cell viability (mean  $\pm$  standard deviation), for 24h exposure, following the WST-8 protocol (Protocol I) \* indicates significant differences between control at  $p < 0.05$ .

Subsequently, there were made some modifications to the experiment, according to Protocol II. Following 24h exposure, the cells were washed with PBS and added complete culture medium with WST-8 to avoid any possible interference from the nanoparticles in the culture medium. The O.D. values were slightly lower than those of the previous readings, however there was no significant changes on cell viability ( $p > 0.05$ ) (Figure 14).



**Fig. 14.** Effects of GNP in A549 cell viability (mean± standard deviation), according to Protocol II.

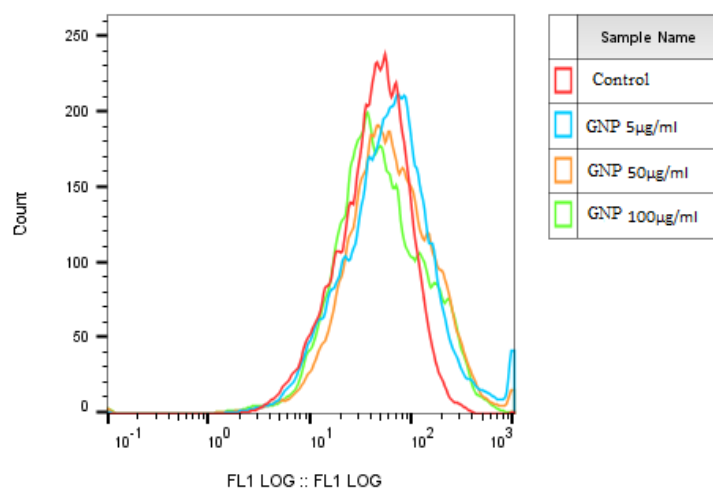
Nanotoxicity can be caused by nutrient depletion induced by the nanoparticles and so the impact of culture medium absorption on the toxicity of GNPs (Protocol III) was tested. For this case, data shows an increase on cell viability ( $p < 0.05$ ) (Figure 15) which can demonstrate that there is no depletion of nutrients by the GNPs and that in turn the medium in contact with the cells is stimulating cell proliferation.



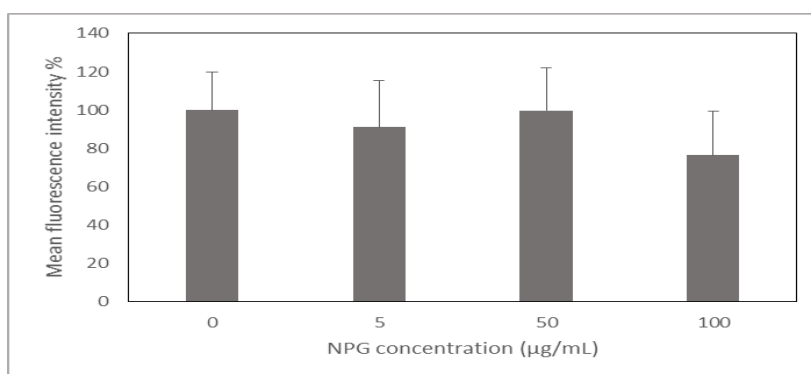
**Fig. 15.** Viability of A549 cells after exposure to F12K medium pre-incubated with GNPs for 24h - Protocol III (mean± standard deviation) \* indicates significant differences between control at  $p < 0.05$ .

#### 6.4. Intracellular ROS detection

To evaluate the ability of GNPs to induce oxidative stress, A549 cells were exposed for 24h with concentrations of 5, 50 and 100  $\mu\text{g/ml}$ . Since there were no changes in cell viability, it was not possible to calculate an inhibitory concentration. For this reason, three concentrations were chosen to continue the studies: a lower concentration (5 $\mu\text{g/ml}$ ), an intermediate concentration (50 $\mu\text{g/ml}$ ) and a higher concentration (100 $\mu\text{g/ml}$ ) (Figure 16). Following the exposure time, cells were analysed by flow cytometry, with DCFH<sub>2</sub>-DA as probe. As shown in Figure 17 there were no significant differences between the exposed concentrations and the control ( $p>0.05$ ).



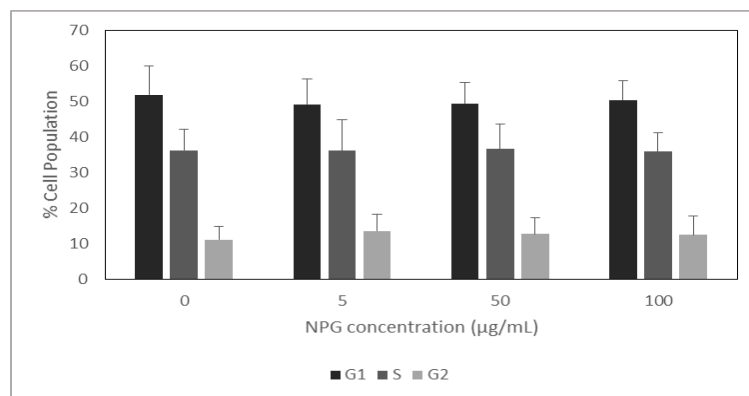
**Fig. 16.** Flow cytometric profile of DCF fluorescence of A549 cells



**Fig. 17.** Intracellular generation of reactive oxygen species (ROS) by A549 cell, at 24 h exposure to GNPs (mean $\pm$  standard deviation).

### 6.5. Cell Cycle Analysis

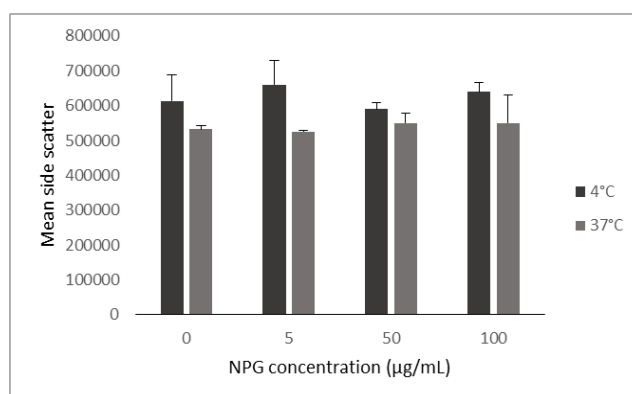
To examine if there were alterations in the cell cycle, cells were exposed for 24h and then collected for analysis by flow cytometry. The results are presented in Figure 18 and show that there is not a significant difference among the cell cycle stages ( $p>0.05$ ).



**Fig. 18.** Cell cycle analysis of A549 after 24h exposure to GNPs. The values are the mean % of cell population ( $\pm$  standard deviation) of the three cell cycle stages.

### 6.6. Cellular Uptake potential of nanoparticles

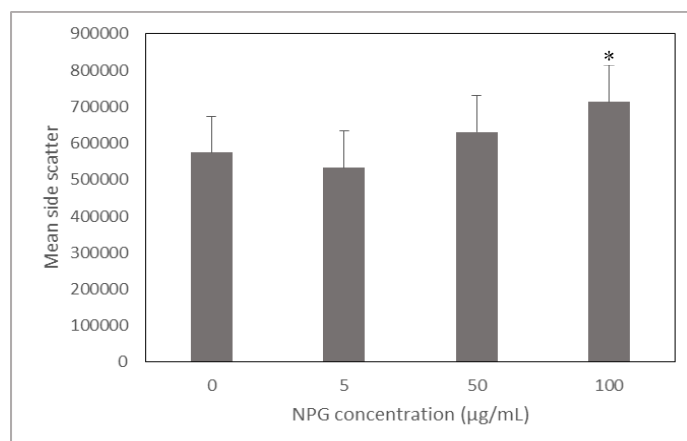
The uptake potential of nanoparticles was assessed using flow cytometry. The side scatter parameter was used to determine the quantitative analysis of the intracellular signal (Figure 21). The first set was incubated for 4h at 37°C and the second set was incubated for 4h at 4°C, for endocytosis inhibition study, with concentrations of 5, 50 and 100µg/ml. The results are shown in Figure 19. There was no significant differences between the control and the exposed cells ( $p>0.05$ ).



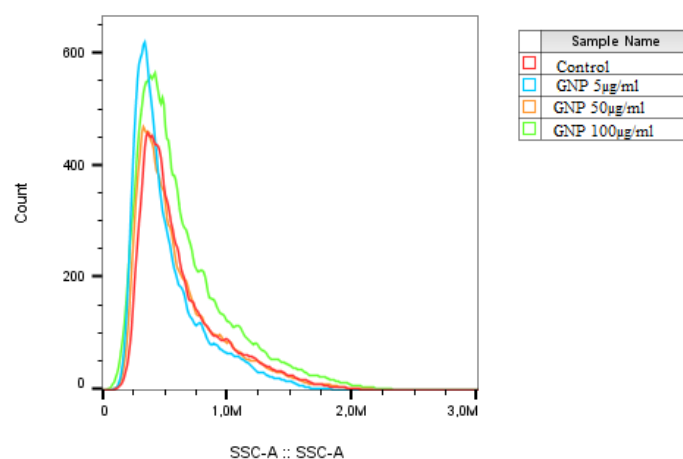
**Fig. 19.** Uptake potential of GNPs by A549 cells after 4h exposure at 4°C and 37°C, assessed by flow cytometry, using the side scattered light (mean $\pm$  standard deviation).



The third set of cells was incubated for 24h, at 37°C, with concentrations of 5, 50 and 100 µg/ml. After the exposure time, there was a slight increase in the values of SS, with a greater significance to 100 µg/ml relative to the control ( $p < 0.05$ ) (Figure 20).



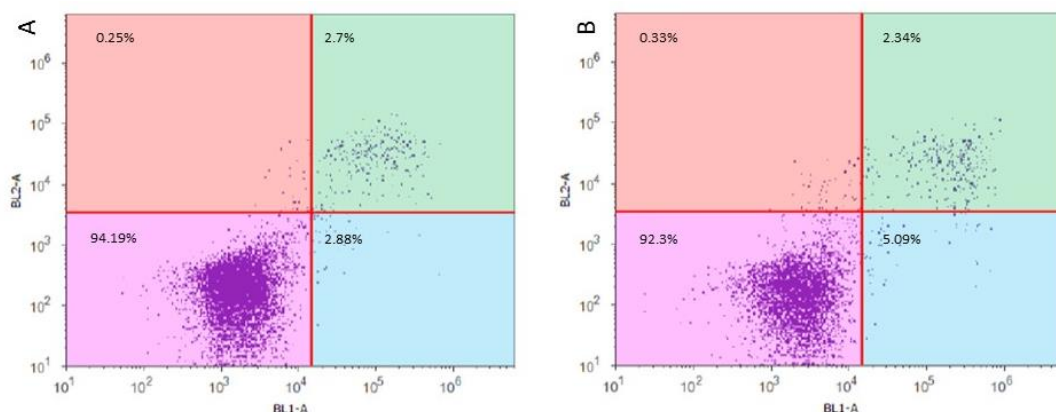
**Fig. 20.** Uptake potential of GNPs by A549 cells after 24h exposure at 37°C, assessed by flow cytometry, using the side scattered light (mean ± standard deviation). \* indicates significant differences between control at  $p < 0.05$ .



**Fig. 21.** Analysis of the uptake of GNPs by flow cytometry light scatter.

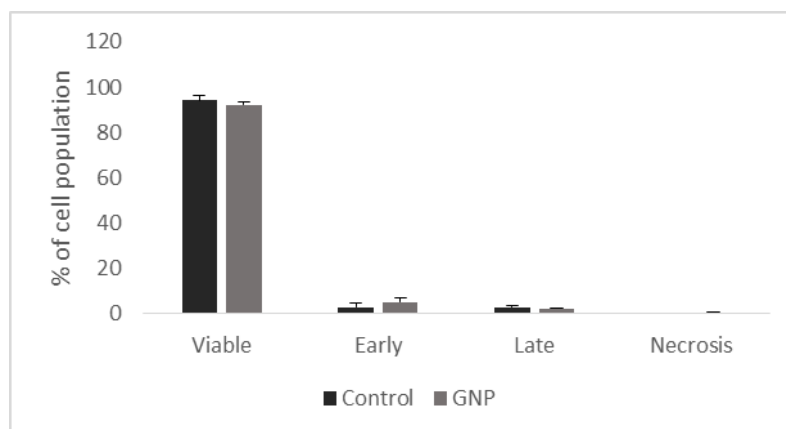
### 6.7. Apoptosis/ Necrosis – Annexin V/PI assay

To measure the apoptotic/necrotic cells ratio, Annexin V/PI assay was used. The measurements were done by flow cytometry using double staining with Annexin V-FITC and PI. This double staining allows the differentiation between early-stage apoptotic cells, late-stage apoptotic cells, necrotic cells and also viable cells (Figure 22).



**Fig. 22.** Apoptosis evaluation by flow cytometry using Annexin V-FITC and PI assay. Scatter diagrams of control (A) and cells exposed to 100µg/ml of GNPs (B).

A549 cells were exposed to the 100µg/ml of graphene nanoplatelets, for 24h. As shown in Figure 23, there were no significant differences in the percentage of cells exposed and the control ( $p > 0.05$ ).



**Fig. 23.** Annexin V-FITC and PI assay for apoptosis assessment of A549 after 24h exposure to GNPs.  $\pm$ SD

## Discussion

The human exposure to nanomaterials can occur through multiple routes, as for instance inhalation, ingestion, skin absorption, injection or implantation, but the major route of human exposure to nanomaterials, such as graphene is through inhalation. Therefore, in the present study the lung cell line A549 was used as a model with the purpose of ascertaining how lung cells can be affected in contact with GNPs.

Graphene nanoplatelets consist of stacked graphene sheets and usually have a thickness between 2-10 nm (Pumera 2009). The characterization results, by DLS, revealed that GNPs exhibited a Z-average diameter of  $268 \pm 6$  nm in complete culture medium. In water was  $268.37 \pm 8.96$  nm and slightly increased when the particles were dispersed in culture medium, which can indicate particle slight agglomeration or the formation of a protein corona. In water the GNPs show a polydispersity index (PdI) of 0.36 indicating moderate polydispersion, while in culture medium the PdI increases to 0.49 indicating a broad polydispersion, which may indicate some degree of agglomeration. This data are also supported by the analysis of the average size distribution that indicates the presence of two peaks, with different intensities. As to the zeta potential, GNPs in water carried a strong negative surface charge ( $\zeta$  -37 mV), while in culture medium the particles surface was considerably less negative ( $\zeta$  -8.44 mV). The less negative zeta potential indicates that the particles are more prone to aggregation.

Lammel et al. (2013) showed that GO and carboxyl graphene nanoplatelets (CXYG) presented different behaviours depending on the supplementation of the culture medium. In less supplemented medium (5% of FBS) within 5 min it occurred flocculation and formation of aggregates. However, when the medium was supplemented with 10% FBS such observations were not found. In another study (Lammel et al. 2014), they used PLHC-1 cell line in culture medium supplemented with 5% FBS and the particles remained dispersed. These observations suggest an optimal concentration of serum between 5-10%, where the NPs are stable in culture medium. Though, in this present study, it was noticed some agglomeration by GNPs on cell exposure, after 24h incubation.

In the present study, cellular viability of A549 was accessed by the WST-8 assay (Protocol I). The WST-8 assay is a highly water soluble tetrazolium salt and it is used as an indicator of cell viability (Tominaga et al. 1999). As described by Tominaga et al. (1999), the WST-8 assay has a higher sensitivity when compared with conventional tetrazolium salts, like

MTT. A significant increase in cell viability was observed for the lowest GNPs concentration (5µg/ml). In a study made by Ahlinder et al. (2015) with A549 cell line, where they examined the toxicity of graphene oxide nanoparticles (GONPs), similar results were found. The exposure of GONPs for 30h showed a dose-dependent increase in fluorescence intensity, and for the lowest concentration (1.56µg/ml) it was observed the most significant difference to the control, regarding an increase on cell viability (Ahlinder et al. 2015). Hu et al. (2011) analysed the interactions between GO nanosheets and cell line A549, reporting that proteins present in the FBS attenuated GO cytotoxicity. A549 cells were treated with GO and incubated in RPMI 1640, supplemented with 10% FBS. When the supernatant was collected, they found that the amount of serum proteins had decreased with the increasing concentration of GO (Hu et al. 2011). These data suggested interactions between GO and FBS proteins. Further analysis showed that when cells were exposed to GO coated with serum proteins, the cell viability was close to 100%. So, they concluded that the serum proteins are capable of reducing the cytotoxicity of GO nanosheets by weakening the interactions between GO and the cells. These facts may present a reason why there was an increase in cell viability in the present study with GNPs.

When culture medium with GNPs was removed and cells were washed before addition of WST-8 reagent (Protocol II), the O.D. values slightly decreased when compared to the values obtained in Protocol I. There were no significant changes in cell viability relative to control, upon exposure to GNPs. Therefore, the influence of GNP on cell viability is not observed. These results show that there is no increase or decrease in cell viability but that there is some interference of the WST-8 reagent and GNPs.

According to Protocol III, the goal of this assay was to verify if the nanoparticles caused nutrient depletion in the culture medium. If the cells did not survive in the medium pre-treated with GNPs, it could be assumed that there was a toxicity caused by the absorption of nutrients by the nanoparticles. However, the results indicate that there is no decrease, but rather an increase in cell viability. These data suggest that in some way, the GNPs stimulate cell proliferation, which has also been reported by others authors (Ruiz et al. 2011; Ahlinder et al. 2015). Chang et al. (2011) performed this same protocol, where the absorption of nutrients was tested by GO in the A549 cell line. Cells exposed to the medium pre-treated with GO showed a proliferation very similar to the control cells. These

results suggest that even if there is nutrient absorption by GO, this does not affect the state of the cells.

Liao et al. (2011) studied the cytotoxicity of graphene oxide (GO) and graphene sheets (GS) by measuring mitochondrial activity in adherent human skin fibroblasts, using the MTT and WST-8 assays. The MTT assay resulted in a false positive signal due to its reduction by GO and GS. Previously studies had been described reporting interferences between MTT assay and carbon-based materials (Monteiro-Riviere et al. 2006; Wörle-Knirsch et al. 2006). In addition, Liao et al. (2011) reported that lactate dehydrogenase was adsorbed by GO and GS, revealing underestimated results. Through this tests, they found that WST-8 would be a good method for evaluate cell viability. However, in the present study, it was found that the WST-8 reagent may interfere with the GNPs.

One of the known mechanisms of nanotoxicity is the formation of reactive oxygen species. ROS caused, directly or indirectly, by nanomaterials that can lead to oxidative DNA damage, resulting in the activation of mechanisms involved in mutagenesis, carcinogenesis and aging related diseases (Fu et al. 2014). ROS production can be influenced by various factors such as shape and size of the NPs, particle surface, tendency to aggregate, among others (Fu et al. 2014). However, and despite all the data available reporting induction of oxidative stress by carbon-based nanomaterials, in this study with A549 cells there were no changes regarding the formation of ROS due to GNPs after 24h exposure, even at a highest concentration (100µg/ml).

According to Chang et al. (2011), A549 cells showed induction of oxidative stress when exposed to GO, even at low concentrations. Of the three GO samples tested (GO mixture: m-GO; GO with smaller size: s-GO and GO with larger size: l-GO), the one that caused the most oxidative stress was s-GO (AFM measurements showed a size of  $160\pm 90\text{nm}$ ; Dh in water – 148nm) (Chang et al. 2011). Production of reactive oxygen species by nanoparticles can cause oxidative damage to DNA. When this happens, the cells trigger their DNA repair mechanisms, or cause cell cycle arrest or even apoptosis (Khanna et al. 2015). The results obtained in this study do not display changes in cell cycle of A549 when exposed to GNPs. Nevertheless, there are some studies that show disturbances in cell cycle induced by the interaction with carbon-based nanomaterials. Park et al. (2015) reported an increase in a dose-dependent manner in subG1 fraction and S phase, when BEAS-2B cells were exposed to GNPs. Increasing in the number of cells in subG1 and S phase indicates

apoptosis, which was verified by changes in the levels of proteins associated with cell death by apoptosis.

The cellular uptake potential of GNPs by A549 cells was evaluated by flow cytometry and only the highest concentration (100µg/ml) showed a significant difference relatively to control, when cells were exposed for 24h at 37°C. This may indicate an uptake of GNPs by A549 cells. Park et al. (2015) observed that GNPs were present in the cytosol of BEAS-2B cells, after 24h exposure. However, Chang et al. (2011) investigated if GO nanoparticles entered A549 cells and the results obtained by TEM did not show nanoparticles inside the cells. Identifying the factor that determine the uptake of NPs by the cells is an important step towards to the determination of the toxicity of those NPs. However, the studies relating to this parameter are few and there is little agreement (Kettler et al. 2014). Nevertheless, the internalization of NPs is influenced by their physicochemical characteristics and also by the conditions under which the experiments are carried out (Kettler et al. 2014). Zhang et al. (2012) analysed the cytotoxicity and cellular uptake of MWCNTs, GO and nanodiamond (ND) on HeLa cell line. The results revealed that all the compounds were internalized by cells, and ND had a higher uptake ratio, followed by MWCNTs and then GO. However, ND induced less cytotoxicity, suggesting that toxicity may not be directly related with cellular uptake of carbon nanomaterials. Kostarelos et al. (2007) also demonstrated that functionalized carbon nanotubes were easily internalized by mammalian and prokaryotic cells. The high aspect ratio and their cylinder shape facilitates the entry through the plasma membrane of cells. Protein-coated graphene oxide nanosheets (PCGO) were observed, upon SEM examination, to be adherent to surface of mouse mesenchymal progenitor C2C12 cells (Mu et al. 2012). By flow cytometry studies (using mean fluorescence intensity), it was demonstrated that the cellular uptake of PCGO was time and size-dependent. Another study (Ahlinder et al. 2015), used confocal Raman spectroscopy to access the interactions between A549 cell line and graphene oxide nanoparticles. They found that the NPs were attached to cells, covering a large surface area and induced small chemical changes.

The externalization of phosphatidylserine (PS) is one of the earliest events in apoptosis. When apoptosis is induced, the inner PS present in the cell membrane is translocated to the outer leaflet of the membrane. The apoptosis assay uses Annexin V to detect PS in the external side of the cell membrane. Propidium iodide (PI) is combined with Annexin V to

stain necrotic cells, since it is an impermeable dye that only enters the cell and intercalates DNA if the cell membrane is compromised. In the present study there was no significant increase in the proportion of apoptotic/necrotic cells after treatment with GNPs. This results show that GNP did not induce apoptosis/necrosis to A549 cells. Similar results were observed by Chang et al. (2011). When A549 were exposed to different sizes of graphene oxide (GO) it did not induced any apoptosis or necrosis. Wang et al. (2014) measured apoptosis on human multiple myeloma cells (RPMI 8226 cells) when exposed to GO (10, 50, 100  $\mu\text{g/ml}$ ) and they found that there were no significant changes in the apoptosis rate after treatment of cells and the control. However, there are other studies with different results. For example, Vallabani et al. (2011) analysed the toxicity of GO in normal human lung cells (BEAS-2B) and they observed a significant increase of early and late apoptosis in exposed cells, when compared to control. Studies (Jaworski et al. 2013) using graphene platelets (GP) (100 $\mu\text{g/ml}$ ) on glioblastoma multiforme cells (U87 and U118) indicated that GP induced apoptosis and necrosis to both cell lines. These different results may be related to the various forms that exist for the synthesis of graphene. In addition, the type of cells analysed may also influence the results.

The *in vivo* studies using graphene nanoplatelets demonstrated nanoparticle accumulation in the lungs, even after 28 days after the first instillation and sub-chronic inflammation in lungs and pleural space (Park et al. 2015; Schinwald et al. 2012). However, there are studies that don't support these results (Kim et al. 2016).

## Conclusions – Future Perspectives

The lungs are the primary route of entry of nanoparticles and so, it is crucial to access the effects of these compounds on their cells. In this study it was evaluated the cytotoxic potential of GNPs on the human lung adenocarcinoma cell line (A549).

GNPs, analysed by DLS, exhibited some degree of agglomeration, which was supported by the values of zeta potential.

The analysis of cell viability by WST-8 assay showed that GNPs did not affect the cell viability of A549 cell line. The cellular uptake of GNPs by A549, assessed by flow cytometry, only showed a significant increase in the values of SS for the highest concentration (100 µg/ml), when cells were exposed for 24h at 37°C. Also, in this study, there were no changes regarding the formation of ROS when A549 were exposed to the GNPs, even at the highest concentration (100 µg/ml). Similar results were observed upon the examination of the cell cycle: there were no changes on cell cycle of A549 when exposed to GNPs. The results indicate that GNPs did not induce apoptosis/necrosis to A549 cells.

In general, it can be concluded that graphene nanoplatelets did not induce cytotoxicity to A549 cell line.

In future work it would be important to study other forms of graphene, with different surface functionalization. Since the lung is a major pathway for the entry of nanoparticles, it would be essential to evaluate the cytotoxicity potential of graphene nanoparticles in other lung cells models, either primary cell lines or established cell lines. Ultimately, *in vivo* testing could be done in order to understand the pathways these NPs go through once inhaled and their interactions with an organism as a whole. Although there are some studies in this regard, the results are still inconclusive.



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